# MEASUREMENT OF EXHALED NITRIC OXIDE AND CARBON DIOXIDE IN THE BREATH OF BEEF CALVES

By

BEN PATRICK HOLLAND

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Thesis Approved:

Clinton R. Krehbiel
Thesis Advisor

David L. Lalman

Anthony W. Confer

A. Gordon Emslie
Dean of the Graduate College

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## NOMENCLATURE

| | |
|---|---|
| 3MEIN | 3-methyleneindolenine |
| 3MI | 3-methyl indole |
| bAM | bovine alveolar macrophage |
| BHV-1 | bovine herpesvirus type 1 |
| BRD | bovine respiratory disease |
| BRSV | bovine respiratory synctial virus |
| BVDV | bovine diarrhea virus |
| cNOS | constitutive nitric oxide synthase |
| EBC | exhaled breath condensate |
| $eCO_2$ | exhaled carbon dioxide |
| eNO | exhaled nitric oxide |
| eNOS | epithelial nitric oxide synthase |
| Hp | haptoglobin |
| IFN-γ | interferon gamma |
| LKT | leukotoxin |
| LPS | lipopolysaccharide |
| iNOS | inducible nitric oxide synthase |
| mRNA | messenger RNA |
| NDIR | non-dispersive infrared |
| NO | nitric oxide |

| | |
|---|---|
| NOS | nitric oxide synthase |
| PI3 | parainfluinza virus type 3 |
| rb | recombinant bovine |
| rh | recombinant human |
| ROS | reactive oxygen species |
| RNS | reactive nitrogen species |
| SaA | serum amyloid-A |
| SPME | solid-phase microextraction |
| TDLAS | tunable diode laser absorption spectroscopy |
| URT | upper respiratory tract |
| VOC | volatile organic compound |

# CHAPTER I

# INTRODUCTION

Bovine respiratory disease (BRD) continues to be a significant and growing problem in the beef industry. The USDA surveyed veterinarians that consult with feedlots in Texas, Kansas, Nebraska, and Idaho from 1994 to 1999 in regard to feedlot mortality (Loneragan et al., 2001a). The results showed, when averaged over time, 12.6 animals died for every 1,000 that entered the feedlot. Of these deaths, 57.1% were attributed to BRD. The death rate from BRD rose significantly during the survey years, but deaths credited to other causes did not increase indicating that current management and treatment strategies have been unsuccessful in controlling BRD. In 1996, for every 1% increase in death loss, cattle feeders lost an estimated $5 to 10 per head marketed (Edwards, 1996). Economic losses related to death loss are not the only results of BRD. Edwards (1996) also reported that around 70% of all disease in Midwestern feedlots is respiratory disease, and for every 10% morbidity rate, medicine costs alone would reach $2 per head marketed. In a 1996 study, 35% of 469 steers were medicated for BRD, but 70% had lesions present on their lungs at slaughter indicating that many cases go untreated (Wittum et al., 1996). These sub-acute cases may have impact on animal growth and performance. Cattle with lung lesions present at slaughter, regardless of whether or not they were treated for BRD, had lower average daily gains, lighter hot

carcass weights, less internal fat, and lower marbling scores than steers without lesions (Gardner et al., 1999). These performance characteristics indicate that BRD may have much more serious economic repercussions than treatment costs and death loss alone. Gardner et al. (1999) also reported that the number of steers treated (n = 102) differed from the number that had pulmonary lesions (n = 87). In multiple studies comparing lesions on the lungs at slaughter to treatment rates in the feedlot, there were treated cattle that did not have lesions, and untreated cattle that did have lesions (Bryant et al., 1996, Wittum et al., 1996a, Gardner et al., 1999). These results may indicate that current methods of diagnosing BRD are inadequate.

Developing an inexpensive and objective method of diagnosing and predicting BRD would be of great benefit to producers. In vitro studies have measured nitric oxide derivatives from bovine immune cells and the enzyme responsible for nitric oxide production in diseased lung tissue (Mason et al., Yoo et al., 1996, 1996, Fligger et al., 1999, Mason et al., 2000). This thesis contains two experiments in which tunable-diode laser absorption spectroscopy (Breathmeter, Ekips Technologies, Norman, OK) was used to measure the concentrations of nitric oxide (NO) and carbon dioxide in bovine breath and test the efficacy of measuring exhaled nitric oxide (eNO) as a clinical biomarker of BRD and(or) the stress that predisposes cattle to become susceptible to BRD.

# CHAPTER II

# REVIEW OF LITERATURE

## Bovine Respiratory Disease

*Pathogens and Stressors.* The complex known as Bovine Respiratory Disease (BRD) is a multifaceted disease that involves both bacterial and viral pathogens and has a variety of risk factors. *Mannheimia* (formerly *Pasteurella*) *haemolytica, Pasteurella multocida, Histophilus somni* (formerly *Haemophilus somnus*), *Mycoplasma bovis,* bovine herpesvirus type 1 (BHV-1), bovine diarrhea virus (BVDV), parainfluenza virus type 3 (PI3), and bovine respiratory synctial virus (BRSV), and bovine respiratory corona virus are common pathogens associated with BRD (Roth and Perino, 1998).

When combined with the stress of weaning and transportation, which has damaging effects on the immune system (Blecha et al., 1984), these pathogens can lead to a high incidence of BRD. The exposure often occurs during shipping and at auction barns where cattle are co-mingled (Galyean et al., 1999). The pathogens involved in the BRD complex work in a synergistic fashion with detrimental results in the host animal. For a more thorough overview of the bovine immune system and BRD, see Griffin (1998) and Roth and Perino (1998).

The principal bacterial agent responsible for the clinical signs and pulmonary pathology associated with BRD is *Mannheimia haemolytica* biotype A, serotype 1

(Whitley et al., 1992). It is hypothesized that management and environmental stress factors and/or viral infection alter the upper respiratory tract (URT) epithelium allowing this serotype, not normally found in the URT of healthy cattle, to colonize. Additionally, *M. haemolytica* possesses several virulence factors including endotoxin (LPS) and leukotoxin (LKT) which enable it to adhere to and colonize respiratory mucosal surfaces, evade host defense mechanisms, and disrupt pulmonary structure and function (Lafleur et al., 2001; Jeyaseelan et al., 2002).

*Current Management Practices.* Upon arrival at a feedlot, cattle can be placed in one of two categories: 1) high-risk and 2) low-risk (Edwards, 1996). High-risk cattle include freshly weaned calves, cattle that have been hauled long distances, cattle that have been assembled at auction markets, and cattle that appear to be highly stressed when they arrive at the feedlot. Low-risk cattle include yearling cattle that came from one source, cattle that arrive from preconditioning operations, and low-stressed calves that have been pre-weaned. In a review of preconditioning studies, Cole (1985) found that preconditioning decreased feedlot morbidity 6% and mortality 0.7%. Nutrition, caused by prior deficiencies and low intake by stressed calves upon arrival, can also impact cattle's susceptibility to BRD (Galyean et al., 1999). Feed intake by lightweight stressed calves averages only 1.5% of body weight during the first 2 weeks after arrival (Galyean and Hubbert, 1995). Upon arrival at a feedyard, cattle are usually vaccinated against many, if not all, of the pathogens that can be part of the BRD complex (USDA-NAHMS, 2000). They can also be vaccinated against *Clostridial* pathogens, treated for internal and external parasites, castrated, and dehorned. Prophylactic antibiotics may also be

administered at this time. One example, tilmicosin phosphate (Micotil), has been shown, when given at processing in receiving trials, to reduce ($P < 0.01$) treatment for BRD (11.9% and 12.9%) compared with controls (43.6%, Galyean et al, 1993). In a study beginning in 1989 at the US Meat Animal Research Center (Clay Center, NE), calves that could be classified "low-risk" received vaccinations against bovine rhinotracheitis, bovine viral diarrhea, and *Pasteurella haemolytica* and were given prophylactic Micotil (Wittum et al., 1996a). After these steps were taken, 29% were treated for BRD and 72% of all animals had lung lesions present at slaughter. Of those that had lung lesions, 70% had never been treated for BRD.

Currently in feedlots, pen riders look at cattle and pull ones they believe to be sick based on subjective criteria (Galyean et al., 1999). Signs, such as lack of attention, rapid breathing, reluctance to move or altered gait, and loss of all nasal discharge can be early indicators of BRD (Deyhle, 1996). The next stages of respiratory illness can result in loss of "fill," lowered ears, and low head carriage with nasal discharge. Cattle, which had previously recovered from BRD, exhibit symptoms much more quickly, because of possible prior lung damage, and must be pulled earlier than cattle who had never been sick before. All these symptoms are very subtle and require skilled personnel to make decisions on which cattle should be treated. These personnel are difficult to find and train. The results of trials in which treatment records were kept and compared to lung lesions at slaughter indicate that the way feedlots diagnose and treat sick cattle are inadequate (Wittum et al., 1996a, Gardner et al., 1999). In both studies more animals had lung lesions at slaughter than the number that was treated, and there were individuals who had never been treated but had lung lesions present at slaughter.

*Objective Methods of Diagnosing Bovine Respiratory Disease.* Both blood solutes and exhaled markers have been researched in hopes of finding an objective method for identifying cattle suffering from respiratory disease. The foremost difficulty with these methods is that the results cannot be obtained quickly or on site.

Acute phase protein concentrations in blood serum seem to have the highest correlation with BRD at the present time (Carter et al., 2002; Berry et al., 2004). Acute phase proteins are produced in the liver as part of an early defense mechanism in response to cellular injury (Eckersall and Conner, 1988). This injury can be a result of infection, inflammation, and advanced malignancies (Saini et al., 1998). In healthy individuals, acute phase proteins are virtually non-existent (Alsemgeest et al., 1994). Research in this area has focused on serum haptoglobin (Hp) and serum amyloid-A (SaA) concentrations (Wittum et al., 1996b; Young et al., 1996; Carter et al., 2002; Berry et al., 2004). Wittum et al. (1996b) found associations between serum Hp levels and respiratory disease but found that Hp alone could not predict clinical disease. However, Carter et al. (2002) and Berry et al. (2004) reported that Hp alone could be used as a diagnostic tool to make management decisions regarding BRD. Berry et al. (2004) showed positive correlations between Hp concentrations sampled at processing and the number of subsequent treatments for signs of BRD. The same report, though, did not find differences in SaA concentrations among steers treated different numbers of times for BRD.

Another compound that has been investigated is 3-methyleneindolenine (3MEIN) and 3MEIN-adduct (Loneragan et al., 2001b; Loneragan et al., 2002). 3MEIN is a toxic metabolite of 3-methyl indole (3MI), which is generated in the rumen by tryptophan

fermentation. Loneragan et al. (2001b) showed greater concentrations of 3MEIN in blood and lungs of cattle with acute interstitial pneumonia that in healthy controls. In a receiving trial, 3MEIN-adduct concentrations in the blood peaked during the period of greatest risk for the development of BRD (Loneragan et al., 2002).

Evidence of oxidative stress is another potential marker. Chirase et al. (2002) indicated that oxidative stress biomarkers could be used to predict BRD susceptibility. They showed that pre-transit concentrations of cellular glutathione peroxidase correlated with BRD episodes at the feedlot. However, no other studies on the subject could be found.

Biomarkers in exhaled breath have also been tested (Reinhold et al., 2000; Spinhirne et al., 2003; Spinhirne et al., 2004). The advantage of breath testing is that it is noninvasive and does not require handling and storing body fluids. Leukotriene B4 increased in exhaled breath condensate (EBC) when exposed to experimental bacterial and viral infections (Reinhold et al., 2000). However, the authors noted that such breath tests may not be useful for routine screening. Another study showed acute bacterial infections and/or pneumonia resulted in increased ammonia in EBC (Reinhold et al., 2002). Morbid steers did not exhibit increased ammonia in peripheral blood, though, indicating local production of ammonia in the lung. Using solid-phase microextraction (SPME), Spinhirne et al. (2003) were able to collect volatile organic compounds (VOC) from cattle breath. After a five to fifteen minute collection time, the SPME extraction fibers were transported to a laboratory for gas-chromatography-mass spectrometry analysis. In 2004, the same group identified 21 VOC in cattle breath (Spinhirne et al., 2004). They were able to associate acetaldehyde and decanol with clinically morbid

steers, while methyl acetate, heptane, octanol, 2,3-butadione, hexanoic acid, and phenol were associated with healthy steers. The length of sampling time and the necessity of laboratory equipment and off-site analysis are limiting factors to using both these methods in a timely manner at a feedlot.

## Nitric Oxide

*Health and Disease of the Respiratory System.* Over the past decade, the role of nitric oxide (NO) in airway physiology and pathology has been studied by numerous researchers. NO is produced by many cell types in the body in a reaction catalyzed by the enzyme nitric oxide synthase (NOS, Ricciardolo et al., 2004). The NO radical diffuses rapidly from the point of synthesis, permeates cell membranes, interacts with intracellular sites on both the generating and target cells, and degrades rapidly. All of these are properties that eliminate the need of extracellular NO receptors or targeted NO degradation. NOS catalyzes the oxidation of L-arginine to NO and L-citrulline. Three isoforms of NOS exist: neuronal NOS (nNOS); epithelial NOS (eNOS); and inducible NOS (iNOS). The constitutive forms of NOS (nNOS and eNOS) have been shown to be modulators of airway and blood vessel tone. However, iNOS is controlled chiefly by transcription, which is initiated by exposure to proinflammatory cytokines, such as interferon gamma (IFNγ) and lipopolysaccharide (LPS; Nathan, 1995). The NO radical diffuses rapidly from the point of synthesis, permeates cell membranes, interacts with intracellular sites on both the generating and target cells, and degrades rapidly. All of these are properties that eliminate the need of extracellular NO receptors or targeted NO

degradation. High levels of NO produced by iNOS have effects in killing tumor cells, halting viral replication, and eliminating other pathogens including *Mycobacterium tuberculosis* (Denis, 1991). Mechanisms may involve inhibition of DNA synthesis by inactivation of ribonucleotide reductase and direct de-amination of DNA (Kwon et al., 1991; Wink et al., 1991); S-nitrosylation of cysteine proteases by NO (Saura et al., 1999); and the subsequent reaction of NO with the superoxide anion ($O_2^-$) to form peroxynitrite ($ONOO^-$; Radi et al., 1991). Peroxynitrite is a strong oxidant and cytotoxic molecule. The stimulation of airway epithelial cell beat frequency in the bovine by isoproterenol, bradykinin, and substance P is dependent on an L-arginine/NO pathway (Jain et al., 1993). Inducible NOS stimulated by macrophage-derived cytokines (TNF-α and IL-1β), enhances this motility as part of the hostdefense mechanism (Jain et al., 1995).

Reactive oxygen species (ROS), generated by various enzymatic reactions and chemical processes or inhaled are important in many physiological reactions and immune function (Ricciardolo et al., 2004). ROS can interact with NO to form other reactive nitrogen species (RNS). However, their presence can result in oxidative stress. Environmental pollutants, infections, inflammatory reactions, or decreased levels of antioxidants can all lead to increased levels of ROS and RNS. These may contribute to the pathogenesis of the virally infected lung (Akaike and Maeda, 2000). Other deleterious effects on airways including damage to DNA, lipids, and carbohydrates can occur and lead to impaired cellular function and enhanced inflammatory reactions. Eiserich et al. (1998) discussed damage by RNS to the respiratory tract including affecting tyrosine residues, thiols, and heme groups, altering lipid oxidation pathways, causing DNA damage, and inhibiting mitochondrial respiration. They can also

compromise cell function leading to both apoptosis and necrosis (Murphy, 1999). All these may negatively affect both the pathogen and the host.

*Nitric Oxide in Ruminant Respiratory Systems.* To date several researchers have investigated the production of NO by ruminant species' cells in vitro, and iNOS has been isolated from infected tissue of cattle that have succumbed to BRD. LPS from pathogens involved in BRD have also been investigated in these studies. In bovine mammary tissues, nitric oxide is produced by mononuclear cells in response to IFNγ alone or in combination with *E. coli* LPS (Boulanger et al., 2001). In response to *Pasteurella multocida* LPS and live *Pasteurella* cells, higher levels of NO was produced by buffalo polymorphonuclear (PMN) cells than non-stimulated PMNs (Roy et al., 1996). Animals that had been vaccinated against hemorrhagic septicemia had even higher levels of NO production upon PMN stimulated. This indicates that vaccination increases the immune response in regards to NO production. Bovine alveolar macrophages (bAMs) have the most relevance to BRD. Mason et al. (1996) stimulated bAMs from lungs with no gross pathology. They measured nitrite (a stable metabolite of NO) in cell free supernatants and iNOS messenger RNA (mRNA). Key findings were that bAMs produce NO in response to LPS in a time and dose dependent manner, recombinant bovine (rb) IFNγ, rb interleukin IL-1β, recombinant human (rh) tumor necrosis factor alpha (TNFα), and LPS enhance NO production in a synergistic fashion. They also found that production of NO by bAMs appears to be regulated by mRNA. Inducible NOS mRNA was maximally expressed at 8 hours after exposure, but was detectable for at least 48 hours. The same year, a similar study at the University of Minnesota also found that TNF, IL-1, IFNγ, and

endotoxin (*Pasteurella haemolytica* LPS) stimulated the production of iNOS (Yoo et al, 1996). However, they were able to measure iNOS mRNA at 2 h with a peak at 24 h. Again, they measured supernatant nitrite to approximate NO production and found nitrite detectable at 1-2 h. The levels markedly increased after 12 h, and the peak was 24 h post stimulation. However, these levels were directly correlated to the amount of bAMs present. This group also experimentally infected calves with *P. haemolytica* and saw nitrite levels in broncheoalveolar lavage fluids rise 3.5-fold, 48 h after inoculation. They concluded that NO has a role in the pathogenesis of lung injury in bovine pneumonic pasteurellosis. In 1999, Fligger et al. evaluated archived lungs of the Institute for Animal Pathology at the University of Berne (Switzerland). Lungs from calves that had succumbed to pneumonia were examined for the expression of iNOS. Lung tissue from animals suffering from bronchopneumonia had iNOS present. *Arcanobacterium pyogenes* infected lungs had cells expressing iNOS in a narrow zone encircling necrotic foci and in less damaged areas of inflamed tissue and by some cells in the supportive cartilage of the bronchi. Bronchopneumonia caused by *Pasteurella haemolytica* was also characterized by the presence of considerable numbers of iNOS expressing cells in the zones surrounding necrotic foci. The frequency of iNOS expressing cells was lower in *P. haemolytica* infected lungs than *A. pyogenes* infected lungs. The expression of iNOS was typically seen at the transition from viable to necrotic tissue. However, it is notable that half the lungs in this survey were from newborn calves and a majority of the remainder was from calves less than six months old. While LPS from bacterial pathogens is shown to stimulate NO production in vivo, BVDV, BHV, and PI3 depress NO production by affecting bAM viability (BVDV and BHV) and altering bAM function (PI3, Mason and

Bochsler, 2000). The results of these studies show that NO has a function in the immune response and pathogenesis related to BRD, and measuring eNO could prove to be a valid biomarker for predicting and diagnosing BRD.

*Exhaled Nitric Oxide.* In humans, all three isoforms of NOS are found in the respiratory tract, and nitric oxide is found in both upper and lower airways (Yates, 2001). In humans, exhaled air contains detectable amounts of NO in the parts per billion range when measured by chemiluminescense (Gustafsson et al., 1991). The measurement of eNO is critically dependent on expiratory flow (Phillips et al., 1996). Guidelines have been established to standardize NO collection in both adults and children (American Thoracic Society, 1999). Exhaled NO levels are determined by: 1) NO production by various cells and in airways and lung parenchyma; 2) diffusion of NO into the capillary circulation; and 3) alveolar ventilation and bronchial airflow (Hyde et al., 1997). The first component, NO production, is dependent on: 1) activity of all three NOS isoforms (De Sanctis et al., 1997; Wechsler et al., 2000); 2) the activity of enzymes which regulate the NOS inhibitor asymmetric dimethyl arginine (Nelin et al., 2002); 3) prokaryotic denitrifying species colonizing the upper and lower airways (Gaston et al., 2002); 4) *S*-nitrosothiol catabolic enzymes (Dweik et al., 2001; Gaston et al., 1998, Snyder et al., 2002); and 5) processes that regulate airway pH and nitrite reduction. Decreased pH in the airways of asthmatics during an attack facilitates the conversion of nitrite to NO (Hunt et al., 2000).

Molecular constituents analyzed in exhaled breath are useful for diagnosing and monitoring multiple human diseases and disorders. *Helicobacter pylori* infection

Table 2.1. Exhaled NO ranges in animals[a]

| Species | eNO Range (ppb) | Number of Reports |
|---|---|---|
| Rabbit | 11.4-24.0 | 8 |
| Guinea Pig | 9.2-16.1 | 5 |
| Rat | 0.7-6.9 | 4 |
| Pig | 5.8-22.0 | 4 |
| Elephant | 27.5-30.9 | 2 |
| Horse | 3.3 | 1 |

[a]adapted from Bernareggi and Cremona (1999)

(Tanahashi et al., 1998), heart transplant rejection (Phillips et al., 2002), and neonatal jaundice (Okuyama et al., 2001) all have clinical breath tests approved by the US Food and Drug Administration (FDA) for diagnosis or monitoring. Recently, exhaled NO (eNO) has been studied in asthmatics compared to healthy individuals. Higher levels of NO are present in exhaled air from asthmatics than healthy individuals (Roller et al., 2002a). In humans, increased eNO levels are also associated with rhinitis, bronchietasis, active pulmonary sarciodosis, antive fibrosing alveolitis, and some pulmonary infections including viral respiratory illness (Ricciardolo et al., 2004). In cats with lower respiratory tract disease, increased eNO was measured compared to healthy cats.

Exhaled NO has also been measured in several animal species. Table 2.1 summarizes reports of eNO measurements in animals (Bernareggi and Cremona, 1999). These measurements were obtained by multiple methods, and some measurements were made while the animal was under anesthesia. No reports in the literature could be found wherein bovine eNO was successfully measured.

Chemiluminescence is the most common technology used to measure eNO in animals and humans. However, chemilunimescense was unable to measure eNO from anesthetized and conscious dairy cattle at a sensitivity of ~1-ppb (Schedin et al., 1997).

Based on this report, if exhaled nitric oxide is to be used as a biomarker of BRD, the technology used must be able to quantify concentrations less than 1 ppb.

*Tunable-Diode Laser Absorption Spectroscopy to Measure Exhaled Nitric Oxide.* High-resolution mid-IR spectroscopy (3-20 μm) is a technique that allows rapid and selective measurements of eNO at concentrations of approximately 1 ppb (Roller et al., 2002b). Some molecules absorb light at a given wavelength. When passing light of this wavelength through a gas cell containing the molecule, only a fraction of the light can be recovered. Using Beer's law, the concentration of the molecule in the gas cell can be calculated. Tunable laser absorption spectroscopy (TDLAS) has been a common method for measuring trace gases with absorption features residing in the mid-IR region of the electromagnetic spectrum. In horses, TDLAS using IV-VI lasers operating at 3.4 μm have been used to show a correlation between exhaled $C_2H_6$ levels and reactive airway obstruction (Skeldon et al., 2005) and exercise induced oxidative stress (Wyse et al., 2005). However, the needs for cryogenic cooling of the lasers and frequent calibrations with calibration gases have limited TDLAS to laboratory settings. The Breathmeter (Ekips Technologies, Norman OK) has incorporated a closed cycle cryogenic refrigerator to eliminate the need for large amounts of liquid nitrogen to maintain cryogenic temperatures. An internal calibration system has also been developed to eliminate the need for calibration with gas standards (Roller et al., 2002 a, b). Exhaled carbon dioxide is measured simultaneously to eNO and customized software calibrates the absorption of both gases minimizing the effects of laser power fluctuations. This allows consistent measurement of eNO concentrations over time and over varying exhalation styles. The

software also has the ability to display eNO concentrations within seconds, and trends in exhaled $CO_2$, $H_2O$, and NO can be viewed instantly. The Breathmeter has been used to measure eNO in both healthy and asthmatic children and adults. To date, approximately 839 individuals have been tested.

When humans' eNO levels are measured with the Breathmeter, they exhale into a mouthpiece for a continuous 15 seconds while the machine is constantly analyzing breath real-time (Roller et al., 2002a). In animals, this is not possible. Instead, breath is collected into a reservoir bag for offline analysis (Roller et al., 2006). Collection of breath for offline eNO measurement in cattle must address: 1) NO production in the nasal and paranasal sinuses; 2) ambient NO contamination (common in feedlot environments where large equipment is burning fossil fuels); 3) a large anatomical dead space; and 4) variable exhalation flow rates.

Attempting to target only lower airway breath during collection reduces contamination by both nasal production and high ambient concentrations, and reducing the exhalation port so that cattle are breathing against constant resistance helps standardize flow of breath across a wide range of exhalation patterns observed in cattle. Monitoring exhaled $CO_2$ has been proposed as a means of standardizing breath collection by partitioning breath in alveolar breath (originating in the lower airways) and anatomical dead space breath (breath resident in the mouth and upper airways). Yu et al. (2004) proposed that end tidal $CO_2$ accounts for some confounding factors involved in breath collection. Such factors include the source of breath (alveolar, end-tidal, deadspace, re-breathed), flow rate, sampling and breath storage technique, and hyperventilation.

Schubert et al. (2001) monitored $CO_2$ in the breath of mechanically ventilated patients and nearly doubled the sensitivity and/or concentration of the target analytes.

In view of the fact that BRD pathology affects lower airways (the region where NO production due to immune challenge is most likely to occur; Mason et al., 1996) and atmospheric NO produced from the combustion of fossil fuels by tractors, trucks, and feed mixers, can contaminate dead space air and confound concentration measurements of eNO in exhaled breath, the bovine version of the Breathmeter is equipped to target lower airway breath for measurement (Roller et al., 2006).

## Summary and Conclusions from the Literature

Bovine respiratory disease is caused by multiple pathogens and stressors. This results in a majority of all feedlot death loss and other economic losses to producers from treatment costs and loss of performance and carcass quality. Currently, consistently recognizing the clinical signs of BRD and successfully identifying and treating sick animals are difficult and subjective.

Several biomarkers found in blood and exhaled breath have been examined with the hope of objectively predicting, diagnosing, and monitoring BRD. However, many of the compounds studied require laboratory analysis that cannot usually be conducted on site or in the amount of time required to make management decisions chute-side.

Nitric oxide, produced by oxidation of L-arginine by one of three nitric oxide synthases, is a compound with many functions in the body and respiratory system. Nitric oxide, produced in large quantities by the inducible nitric oxide synthase as a component of the immune response, has effects in killing tumor cells, halting viral replication, and

eliminating other pathogens. In bovine, iNOS exists in alveolar macrophages, and NO derivatives have been measured in vitro in response to antibodies and BRD pathogen lipopolysaccharide.

Exhaled nitric oxide has been measured in humans and several animal species with increased concentrations being linked to diseases such as *H. pylori* infection and asthma in humans. While researchers have been unsuccessful in attempting to measure eNO from cattle using chemiluminescence, Tunable Diode Laser Absorption Spectroscopy, using an internal calibration system, can measure the low levels (< 1 ppb) observed in bovine breath. The purpose of the study herein was to measure eNO in the breath of both clinically healthy calves and calves exhibiting signs of BRD from both natural and artificial challenge and to correlate eNO levels at arrival and upon treatment to number of subsequent treatments resulting in an objective measure of the bovine immune response.

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# CHAPTER III

# PRELIMINARY CHALLENGE STUDY

## Introduction

A small proof of principle study was initially conducted at the Oklahoma State University Nutrition and Physiology Research Center (NPRC) to validate the performance of the Ekips Breathmeter utilizing TDLAS when measuring eNO and $eCO_2$ in the breath of clinically healthy calves and calves showing signs of BRD resulting from intrabronchial challenge with *Mannheimia haemolytica.*

## Materials and Methods

*Cattle and Management.* Three Angus and Angus-Hereford steers (average initial BW = 251.8 kg) were obtained from the Oklahoma State University research herd and delivered to the NPRC during July 2005 assuring minimum exposure to the pathogens and stressors associated with BRD. Nasal swabs taken prior to initiating experimental treatments indicated no infection with the major bacteria associated with BRD. Treatments included challenged (intra-tracheal *Mannheimia haemolytica* serotype 1; [2 steers]) and non-challenged (1 steer). Throughout the trial, calves were housed in metabolism stalls and fed a diet formulated to meet or exceed NRC (1996) recommendations and given free access to water.

*Mannheimia haemolytica Challenge.* Intrabronchial challenge was done by the technique of Corstvet et al., (1982) with slight modification. Calves were restrained by halter with their heads extended and elevated to minimize the angle between the nares and larynx. A 244 cm long silicone broncho-alveolar lavage catheter (11 mm outside diameter x 3 mm inside diameter, Bivonar Equine Broncho-Alveolar Lavage Catheter, Bivona Medical Technologies, Gary, IN) was passed through each animal's nares, past the nasopharynx and larynx, and into the trachea. At the bifurcation, the catheter was rotated one-quarter to one-half of a turn to facilitate it's advancement into one of the main stem bronchi. The catheter was advanced into the distal airway, which was approximately 238 cm past the external nares. The cuff was inflated with 6 to 10 mL of air to form a seal in the distal airway. Cattle were challenged by intro-airway installation of *M. haemolytica.* The challenge inoculum consisted of 10 mL of $10^9$ CFU per mL of *M. haemolytica* S1 Oklahoma Strain followed by 60 mL of phosphate buffered saline (PBS) to insure that the entire challenge dose was flushed from the catheter.

The challenge material was *M. haemolytica* S1 Oklahoma Strain New (repassed through 3 calves in 2004). Frozen stock cultures were plated onto brain-heart infusion (BHI) blood agar (5% sheep blood) and grown at 37º C in 5% $CO_2$. An isolated colony was propagated in 10 mL BHI broth with rotatory shaking at 120 oscillations/min. at 37º C for 18 h. One hundred µL of suspension was added to 1 L of BHI broth and grown for 6 h. The bacteria were re-suspended in PBS and adjusted spectrophotometrically to a final concentration of approximately 1.0 x $10^9$ cfu/mL (optical density of A600 = 0.65). The non-challenged (placebo) steer was given 70 mL of PBS in place of live culture.

*Sample Collection.* Fans were set up in the metabolism room to create a constant airflow, such that the possibility of airborne pathogens moving from the challenged animals to non-challenged animal was minimized. Approximately 1 h prior to administration of the challenge, a whole blood sample (10 mL Vacutainer with no additive; Becton Dickinson, Franklin Lakes, NJ) for serum haptoglobin (Hp) analysis was collected via jugular venipuncture from each calf. Subsequent blood samples were similarly collected 12 h post challenge and daily for 4 consecutive days, d 8 and d 13. Rectal temperature was measured with an electric digital thermometer (GLA Agricultural Electronincs, San Luis Obispo, CA) prior to challenge and at 3 to six h intervals for two days following challenge, and any observations of clinical signs of BRD were noted. Breath was also collected for eNO and $eCO_2$ measurements at these times.

*Breath Collection Mask and Procedure.* Three identical masks (one per each steer) were constructed for collection of exhaled breath for NO measurement. These masks were similar in design to devices reported by Spinhirne et al. (2003 and 2004). However, their design employed the use of solid phase microextraction to measure exhaled components, while the present device allows real-time sampling by the Breathmeter. A 4.8 L plastic bucket (Fisher Scientific, Pittsburgh, PA) had five holes (approximately 2.54 cm diameter each) cut for attachment of inlet and outlet fixtures. A single hole was cut in the center of the base for the outlet, and four were cut at 90º intervals on the side 2 cm from the base. Male and female 1.9 cm CPVC pipe adapters were connected and sealed with teflon tape through all holes. Gaskets (6 cm outer diameter, 2.5 inner diameter) were cut from rubber inner tube and were held in place on

each side of the hole by the pipe adapters and 2.5 cm galvanized steel washers. Four unidirectional tee connectors (Qosina Corp., Edgewood, NY), with two flutter valves each inverted so air could only enter the mask were attached to the fixtures on the side of the bucket. The outlet fitting was connected to a bacterial and viral filter. The open end of the bucket was covered with a membrane cut from rubber inner tube and attached by wire. An opening approximately (10-12 cm in diameter) was cut in the center of the membrane to allow the mask to fit securely over the animal's nose and mouth.

Initially, prior to the challenge, we attempted to measure breath real time by using constant flow to draw air into the gas cell of the TDLAS instrument. After multiple unsuccessful attempts at measuring NO in exhaled bovine air, we chose to use side-stream offline sampling into a sample bag (Roller et al., 2006). The outlet fixture of the mask was equipped with a $CO_2$ sensor (Capnostat 5, Respironics, Inc.) and a differential pressure sensor. The mask was placed over the mouth and nose of the calves for breath testing. When the $CO_2$ concentration in the air exhaled by the calves exceeded a certain value, a sampling valve opened and a portion of the breath was drawn into an evacuated sample bag kept in a vacuum chamber. The sampling valve was closed by a drop in the pressure which occurs instantly when the exhalation ceased. The higher concentration of $CO_2$ remained slightly longer than the high pressure. By using this method, the air which accumulates in the upper airways, mouth, and the mask's dead space is eliminated before the sample is drawn into the sampling bag, and a sample of entirely lower-airway air is collected. The mask was connected to the instrument by a 0.64 cm inside diameter Teflon tubing approximately 18.5 m in length. Usually, 15 to 25 breaths were required

before achieving the desired sample volume and the mask could be removed. The sample was then pumped into the TDLAS instrument's gas cell for NO and $CO_2$ measurement.

*Haptoglobin Analysis.* Samples collected for serum Hp analysis were allowed to clot overnight at 4ºC, centrifuged, and stored at -10ºC until laboratory analysis could be conducted. Serum Hp concentrations were determined using bovine serum haptoglobin radial immunodiffusion kits (code No. P0105-1, Cardiotech Svcs., Inc., Louisville, KY). Chapter four of this thesis contains a more detailed description of the procedure.

## Results

The two challenged calves showed only mild signs of clinical illness, including increased body temperature, decreased appetite, and depression. At 6 and 24 h after the challenge, average body temperature 41.4ºC vs. 40.4ºC and 40.4ºC vs. 39.4ºC for challenged vs. the control steer respectively (Figure 3.1). The control calf did have higher than normal body temperature which was not completely unexpected given the hot, humid conditions (ambient temperature > 32ºC) on the day of challenge. Serum Hp concentrations were less than the lower standard and could not be measured using the current analysis. Therefore, they were considered to be less than 10 μg/mL which is normal in healthy cattle. Serum Hp levels increased dramatically in both of the challenged steers within 24 hours of challenge, while peak Hp levels occurred > 30 and 48 h post-challenge, respectively (Figure 3.2). Prior to challenge, eNO levels measured with TDLAS (Breathmeter, Ekips Technologies, Norman, OK) averaged 1.13 ± 0.23 ppb for all three steers and $eCO_2$ levels averaged 2.30 ± 0.07%. Maximum eNO levels of

3.02 and 3.00 ppb were measured from the challenged calves at 17 and 25 h post challenge, respectively (Figure 3.3). Approximately 20 h post challenge, $eCO_2$ peaked for all three calves (Figure 3.4).

## Discussion

The primary objective for this study was met in that eNO was measured from the breath of cattle with TDLAS. Based on the rectal temperature and increase in Hp concentrations, along with the observations of mild depression and decreased appetite, the experimental challenge resulted in inflammation and an activated immune system. The time frame associated with the Hp increase after *M haemolytica* challenge was similar to that reported by Conner et al. (1989). The small number of calves used (n=3) did not allow any statistical models to be applied to the data, and any specific conclusions would be premature. However, the observed eNO values suggest that BRD may be associated with higher production and exhalation of NO, which concurs with the in vitro studies of Yoo et al. (1996), Mason et al. (1996), and Mason et al. (2000), and the pathology reported by Fligger et al. (1999). The fact that eNO was measured in the breath of calves, and at higher levels from calves challenged with *M. haemolytica,* along with the evidence of the literature justified conducting a larger study in which both clinically healthy calves and calves exhibiting signs of BRD from natural challenge would have their breath measured with TDLAS.

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Figure 3.1. Rectal temperature of control and challenged steers.

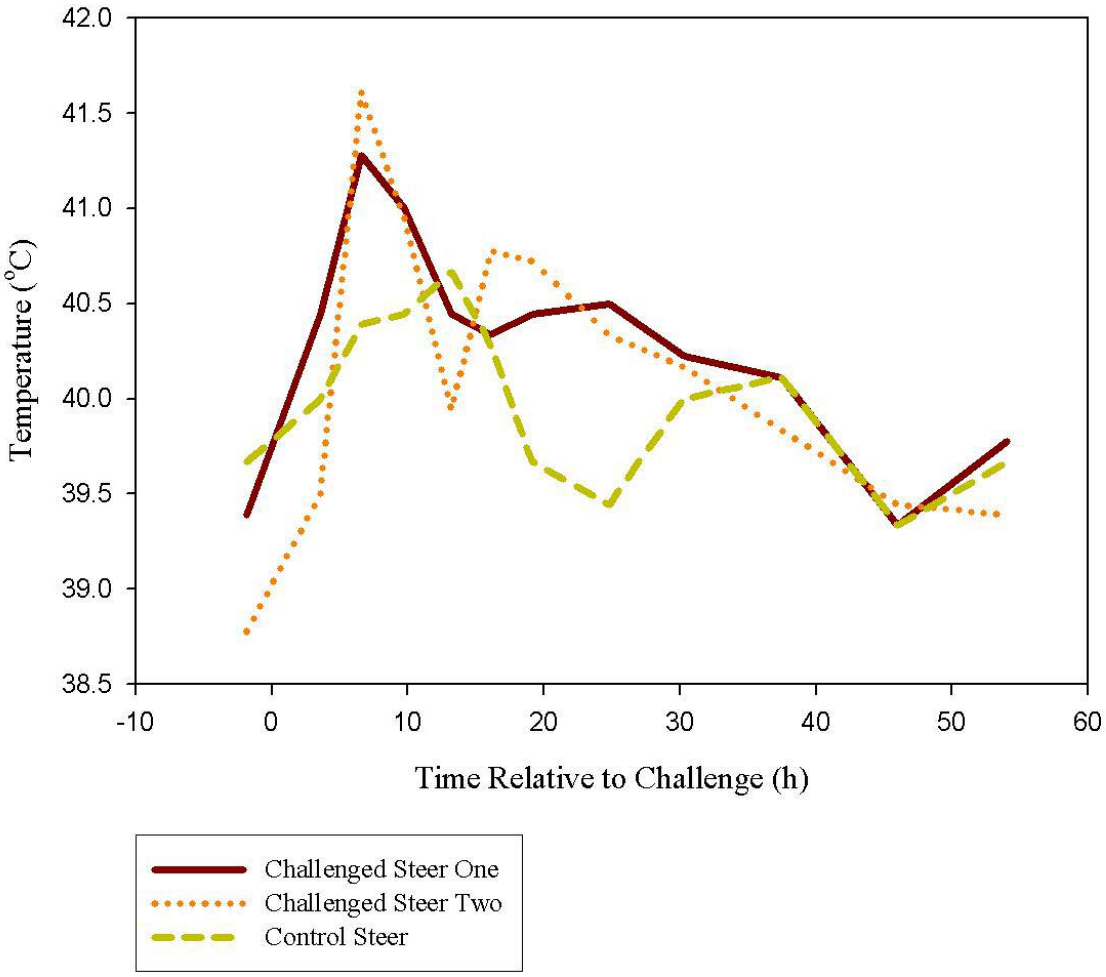


Figure 3.2. Serum haptoglobin concentrations in control and challenged steers.

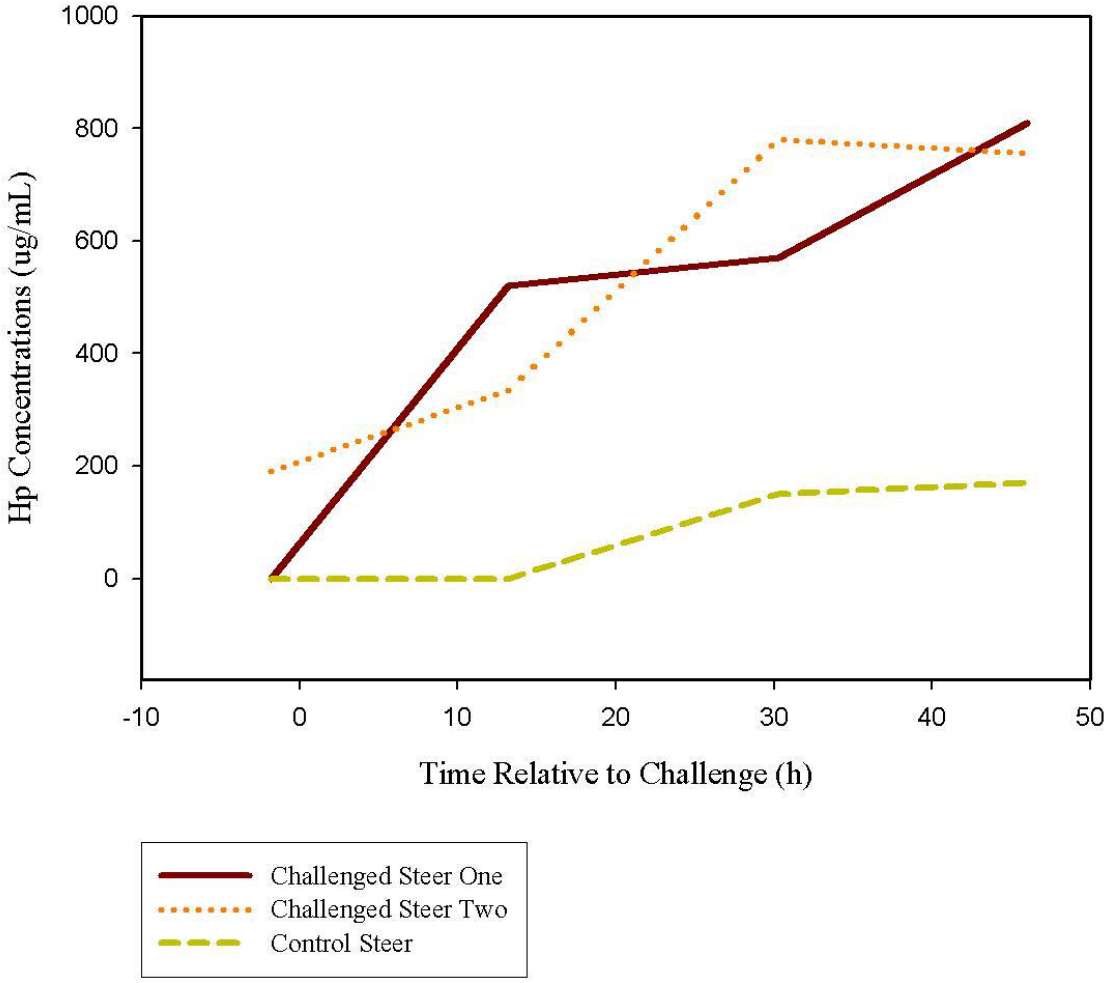


Figure 3.3. Exhaled nitric oxide concentrations in control and challenged steers.

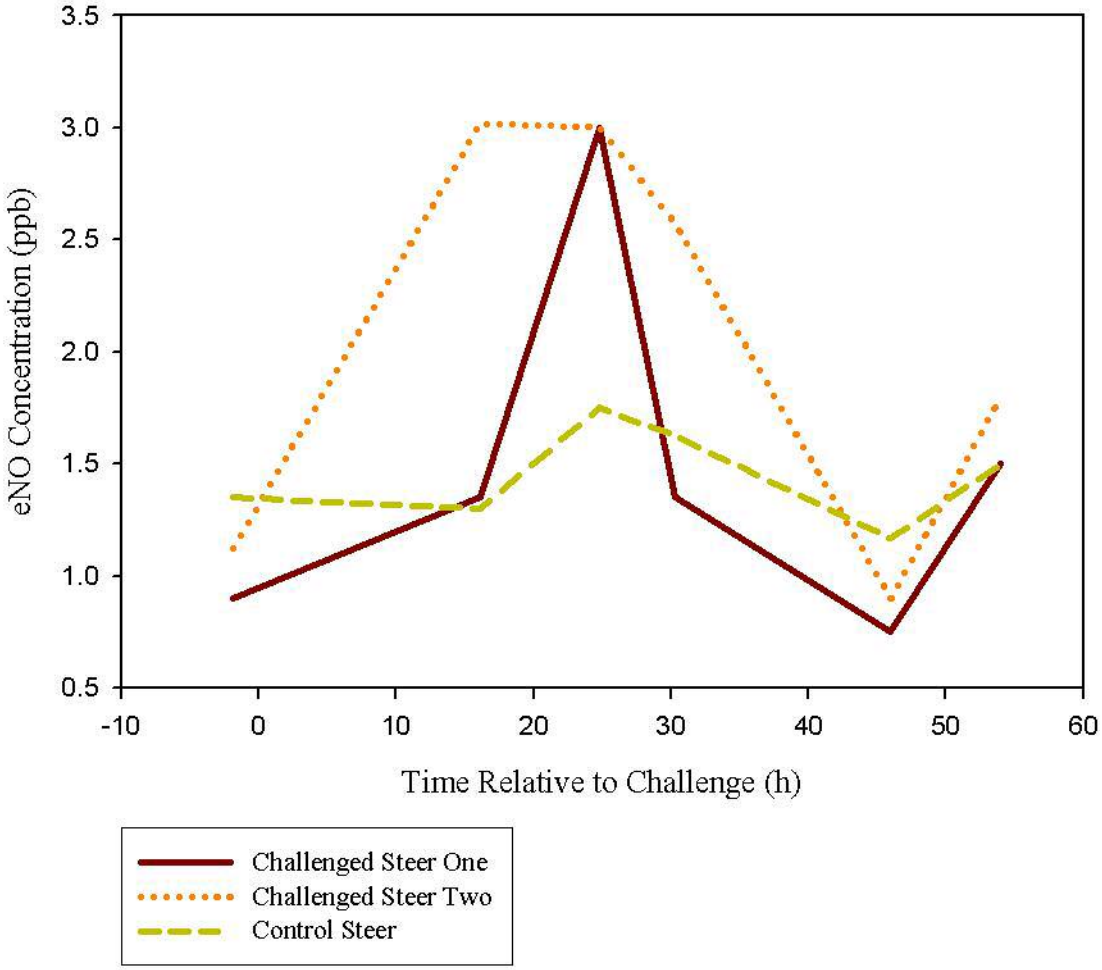
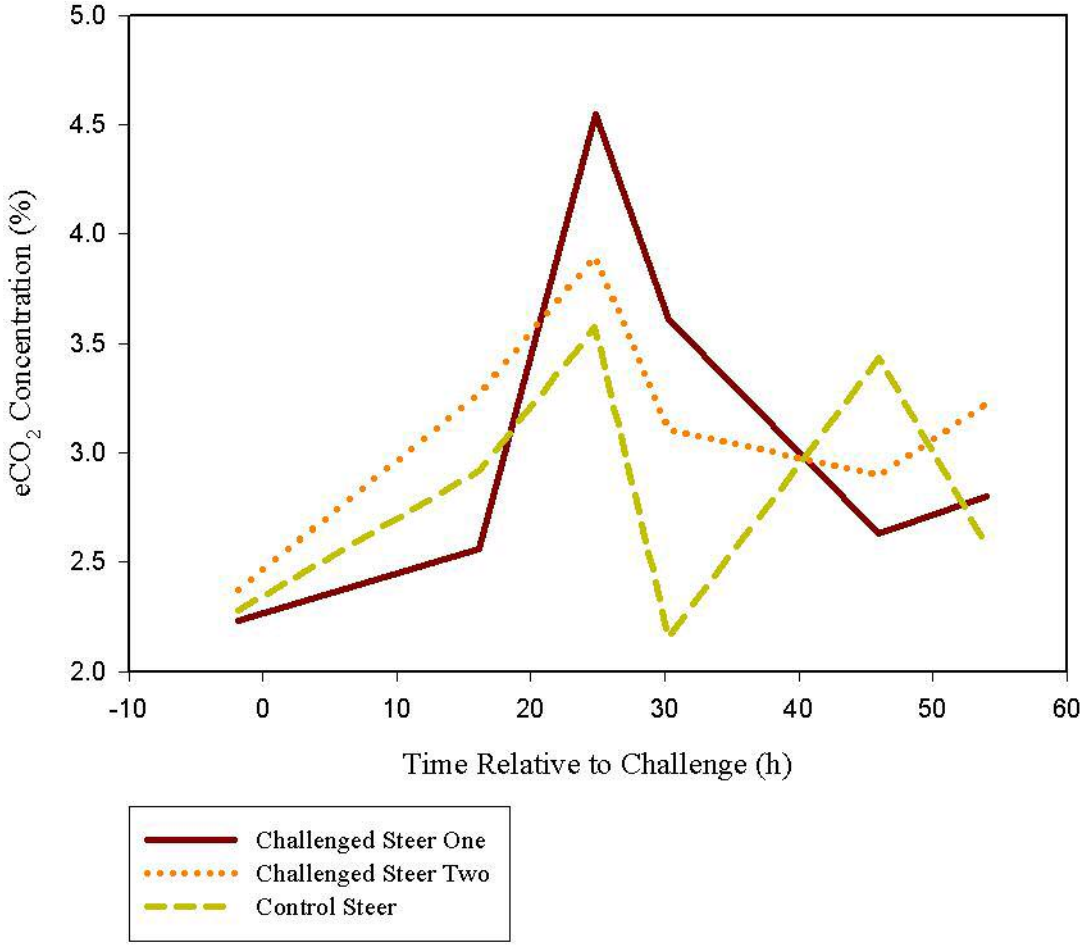


Figure 3.4. Exhaled carbon dioxide concentrations in control and challenged steers



# CHAPTER IV

# MEASUREMENT OF EXHALED NITRIC OXIDE AND CARBON DIOXIDE IN THE BREATH OF NEWLY RECEIVED STEERS


## Abstract

*Objective.* The objective of this study was to measure exhaled nitric oxide and exhaled carbon dioxide using tunable diode laser absorption spectroscopy in the breath of newly received high-risk beef calves.

*Animals.* 395 steer calves (avg. initial BW = 218.6 ± 22.4 kg).

*Procedures.* Upon arrival, and throughout the receiving period, calves had breath collected for exhaled nitric oxide (eNO) and exhaled carbon dioxide (eCO2) measurement using tunable diode laser absorption spectroscopy. All animals pulled and treated for signs of BRD were sampled, and daily, one clinically healthy calf was pulled at random from 50% of the pens for control measurements. Each time an animal's breath was collected, a whole blood sample for serum haptoglobin (Hp) analysis was also collected.

*Results.* Mean eNO upon arrival was 313.9 ± 415.2 ppt and mean eCO2 was 2.64 ± 0.94%. There were no differences between arrival eNO or Hp concentrations and eventual treatment for BRD. However, eCO2 was less ($P < 0.03$) for steers never treated than for those that were treated for BRD. At time of treatment, eCO2 values were higher for control steers than those that were treated. Upon first treatment for BRD, treated animals had higher ($P < 0.05$) eNO than controls.

*Conclusions and Clinical Relevance.* Nitric oxide and carbon dioxide were measured in the exhaled breath of calves. Numeric trends suggest eNO may be useful in diagnosing and monitoring respiratory disease in cattle. Current technology does not allow measurements at the precision required for a clinical test.

## Introduction

Endogenous nitric oxide (NO) is produced in many tissues from the amino acid L-arginine by a group of nitric oxide synthase enzymes (NOS; Ricciardolo et al., 2004). The constitutive and epithelial forms of NOS serve as modulators of airway and blood vessel tone. However, inducible NOS (iNOS) is initiated by proinflammatory cytokines and controlled chiefly by transcription (Nathan, 1995). Nitric oxide is produced in large amounts by when iNOS is present, and NO serve functions in the immune system, such as killing tumor cells, halting viral replication, and eliminating other pathogens. In the bovine, TNF-α and IL-1β produced by alveolar macrophages stimulates airway epithelial motility (Jain et al., 1995). Bovine alveolar macrophages, when cultured in vitro, have produced stable nitric oxide derivatives (nitrite and nitrate) in response to *P. haemolytica*

lipopolysaccharide in a time and dose dependent manner (Mason et al., 1996). The same study showed that multiple cytokines enhanced NO production in a synergistic fashion. In a similar study, Yoo et al. (1996) measured peak iNOS mRNA at 24 h post cytokine and *P. haemolytica* endotoxin challenge and measurable nitrite levels in cell supernatant 2 h post challenge with a marked increase from 12 to 24 h. These researchers concluded that NO has a role in the pathogenesis of lung injury in bovine pneumonic pasteurellosis.

In humans, exhaled air contains detectable levels of nitric oxide in the parts per billion range (Gutaffson et al., 1991). Bernareggi and Cremona (1999) summarized reports of eNO measurements in multiple animal species. However, no reports could be found in the literature in which eNO was successfully measured in cattle. In humans, increased levels of eNO are associated with several illnesses and medical conditions (Ricciardolo et al., 2004). Roller et al. (2002) used tunable diode laser absorption spectroscopy (TDLAS) to measure eNO in humans. They reported that asthmatics exhaled higher levels than healthy individuals. Using chemiluminescence, Schedin et al. (1997) were unable to measure eNO from conscious and anesthetized dairy cattle at a detection limit of ~ 1 ppb. TDLAS (Ekips Breathmeter, Ekips Technologies, Norman, OK), equipped with internal calibration and a background subtraction routine, can measure the lower concentrations (< 1ppb) of NO necessary for eNO measurement in cattle (Roller et al., 2006).

The objective of this study was to measure NO in the breath of newly received beef calves using TDLAS and attempt to correlate eNO concentrations with occurrences of clinical BRD and objective measures of BRD. The work cited above supports the hypothesis that cattle experiencing respiratory disease challenges have activated iNOS

and increased production of NO. Similarly to humans, this NO may be exhaled, and clinically ill cattle could exhale higher levels of NO than healthy calves.

## Materials and Methods

*Cattle and Experimental Design.* Three hundred ninety-five steer and bull calves (mean initial body weight [BW] averaged across four loads = 218.6 ± 22.4 kg) were delivered to the Oklahoma State University Willard Sparks Beef Research Center from central Oklahoma auction markets in September 2005 for a 42-day receiving trial. Approximately 1 h after arrival, calves were individually weighed and tagged in the left ear with a sequential numbered ear tag. A whole blood sample (10-mL Vacutainer with no additive; Becton Dickinson, Franklin Lakes, NJ) for serum haptoglobin (Hp) analysis was collected via jugular venipuncture from each calf, as well as a breath sample for eNO and $eCO_2$ determination was collected. Approximately 36 (loads 1-3) or 72 (load 4) h following arrival, steers were processed. This included recording an individual weight, administering a viral respiratory vaccine (Bovishield Gold 5, Pfizer Animal Health, Exton, PA), clostridial bacterin/toxiod (Ultrachoice 7, Pfizer Animal Health), and dewormer (Ivomec Plus, Merial, Duluth, GA), and castration and dehorning when necessary. Cattle were blocked by weight and allocated into 24 soil surfaced pens (12.2m x 30.5m). Pens had fence line feed bunks (12.2m) with concrete extending from the bunk 3.7 m. Adjacent pens shared fence-line automatic water basins. Throughout the trial, cattle were fed a 60% concentrate receiving ration (Table 4.1). Feed was delivered at approximately 0730 and 1330 daily. In the morning, when bunks were evaluated, if there was no feed remaining, the feed delivery was increased 0.22 kg/animal.

Each morning, cattle were evaluated by trained personnel for signs of BRD. Clinical signs included depression, lack of fill compared with penmates, cough, altered gait or stance, ocular or nasal discharge, or general physical weakness. Calves exhibiting clinical signs of BRD were objectively evaluated by measuring rectal temperature (GLA Agricultural Electronincs, San Luis Obispo, CA). An animal with a rectal temperature greater than 40.0ºC was considered morbid and treated with antimicrobials.

The treatment protocol consisted of a possibility of three antimicrobials being administered. When a steer met treatment criteria the first time **(1st Treatment)**, tilmicosin phosphate (Micotil, Elanco Animal Health, Indianapolis, IN) was administered at a rate of 10 mg/kg BW. If, after a period of at least 72 h, treatment criteria was again met **(2nd Treatment)** enrofloxicin (Baytril 100, Bayer Animal Health, Shawnee Mission, KS) was administered at a rate of 10 mg/kg BW. When an animal met treatment criteria a third time **(3rd Treatment)**, ceftiofur hydrochloride (Excenel RTU, Pharmacia Upjohn, Kalamazoo, MI) at 2.2 mg/kg BW was given 48 h after the second treatment and was repeated in 48 hours. An additional injection of Excenel was also given 48-72 h later if deemed necessary. All animals treated for BRD had whole blood collected for serum Hp determination and breath collected for eNO and $eCO_2$ analysis. In addition, one clinically normal calf was selected daily for the first 28 days of the trial from one half of the pens to serve as a control for blood and breath comparisons. From each pen, only calves that arrived as steers were used as controls to eliminate the possibility of increased serum Hp concentrations in recently castrated steers. Prior to initiation of the trial, steers in each pen were randomized for use as control animals. Care was taken so that no calf that had previously been treated for BRD was used as a control. When all the eligible

steers (did not arrive as a bull and had not been treated for signs of BRD) in a pen had been used as controls, they were re-sampled using the same order. On days 14, 28, and 42 of the trial all cattle were individually weighed, and six animals from each pen had breath and blood collected for eNO, $eCO_2$, and serum Hp analysis as described above. These animals were randomly selected from the calves that arrived as steers from each pen as before. However, treatment for signs of BRD did not exclude sampling of these steers.

On day 41, pens were delivered half the previous day's allotment of feed, and water basins were shut off at approximately 1730 hours. Therefore, any effects of gut fill were minimized for the final weight taken on the following morning.

*Breath Analysis.* Breath was simultaneously analyzed for eNO and $eCO_2$ using TDLAS (Ekips Breathmeter, Ekips Technologies, Norman, OK; Figure 4.1). The components of the Breathmeter as adapted for this study and the methods of breath data analysis are described (Roller et al., 2006). Briefly, a custom made mask (Trudell Medical International, ON, Canada) equipped with one-way flutter valves was placed over the calf's muzzle while restrained in the chute. The mask was equipped with an open-path non-dispersive infrared (NDIR) capnograph $CO_2$ (Capnostat 5, Respironics, Inc., Carlsbad, CA) and differential pressure sensor which engaged a sampling valve based on $eCO_2$ level and a drop in peak exhalation pressure (Figure 4.2). This system enabled standardized side-stream breath sampling across a wide range of bovine exhalation patterns, ensured a relatively consistent sample of lower airway breath was

captured for analysis, and minimized sampling of ambient air. Typically, twenty exhalations were required, which took approximately 20 to 40 seconds. Breath analysis included a background subtraction technique, in which the sample was compared with nitric oxide scrubbed air (Triple Blend, Purafil, Inc., Doraville, GA) in order to limit the low frequency background noise in the measured spectra and allow for the detection of the low concentrations (< 1 ppb) of nitric oxide present in healthy cattle (Figure 4.3).

*Haptoglobin Analysis.* Blood samples for serum Hp analysis were allowed to clot overnight at 4ºC, centrifuged at 2,000 × g for 15 min., and stored at -10ºC until laboratory analysis could be conducted. Serum Hp concentrations were determined using bovine serum haptoglobin radial immunodiffusion kits (code No. P0105-1, Cardiotech Svcs., Inc., Louisville, KY). The procedures were derived and validated by Connor et al., (1989), Makimura and Usui (1990), and Skinner et al. (1991). Serum samples (100 µL) were treated with an equal amount of 40 mM solution of L-cysteine (24 mg of L-cysteine dissolved in 5 mL of L-cysteine solvent [1-e]) and mixed. Two standards, 500 µL/mL and 125 µL/mL bovine Hp, respectively, were added to two specified wells on the test plate. One treated sample was loaded in each additional well of the plate (n=8). The plate was then covered and incubated at 37ºF for 48 h. Following incubation, the external diameter of each precipitin ring was measured with a supplied plastic scale. The results were plotted on the vertical axis of a semilogrithmic graph and Hp concentration was determined from the horizontal axis. A two-fold dilution factor was used to plot the reference curve for each plate using the two standards. Absence of a precipitin ring indicated Hp concentrations less than 10 µL/mL, which is normal. The coefficient of variation was less than 4% for repeated measurements on the same specimen.

*Statistical Analysis.* Data were analyzed using the Mixed Procedure of SAS (SAS Institute, Cary, NC). Individual animal was the experimental unit. For arrival data (BW, ADG, eNO, $eCO_2$, and Hp) the number of treatments for BRD **(Trt0, Trt1, Trt>1)** was considered a fixed effect. Load and weight block were considered random effects. For data collected when animals were treated for clinical signs of BRD, treatment number **(Control, 1st Treatment, 2nd Treatment, 3rd Treatment)** was considered a fixed effect with load and block considered to be random effects. Least squares means were separated using least significant difference. Control data were also contrasted with the average of all treatment data for each response variable. Data from breath tests were eliminated from the analysis if eNO concentrations were above 1.7 ppb indicating contamination from ambient air, the sampling valve was open for less than 5 seconds or greater than 12 seconds, resulting from a sampling valve malfunction, or measured $eCO_2$ concentrations fell below 0.5%, indicating an invalid test.

## Results

Upon arrival, mean eNO was 313.9 ± 415.2 ppt and mean $eCO_2$ was 2.64 ± 0.94% (n=379). During the trial, 56.0% of steers were never treated for signs of BRD **(Trt0)**, 33.9% of steers were treated only once **(Trt1)**, and 10.1% were treated more than once **(Trt>1)**. Table 4.2 lists steer performance and arrival breath values for each of the three groups **(Trt0, Trt1, and Trt>1)**. One steer died from BRD, and two steers died from reasons unrelated to the study. While there were no differences in arrival eNO between steers that never received treatment for BRD and those that did (P = 0.76), eCO2 was less

(P = 0.03) for Trt0 (2.30%) than Trt1 and Trt>1 (average = 2.50%). Arrival Hp did not differ between number of eventual treatments (P = 0.38).

For data collected at the time of treatment for BRD or control, rectal temperature and serum Hp were greater for the average of treated vs. control cattle (P < 0.01). In contrast to arrival day data, control steers had higher (P < 0.0001) eCO2 than steers that were treated for BRD (Table 4.3). Least squares means for eNO values were 368.3 ppt for **Control** steers, 564.5 ppt for **1st Treatment** steers, 369.4 ppt for **2nd Treatment** steers, and 462.2 ppt for **3rd Treatment** steers. The F statistic for the main eNO model was significant (P < 0.05); however, the contrast between control and treated steers was not significant (P = 0.29). When means were separated by least significant difference, eNO least squares means from C**ontrol** vs. **1st Treatment** steers were different (P < 0.01).

## Discussion

*Breath Collection and Measurement.* Using TDLAS was successfully incorporated into the daily activities of the research facility (Figures 4.4 and 4.5). However, additional personnel were required during processing to operate the Breathmeter and hold the mask on the animal during collection. Initially, some animals rejected the mask and resisted its placement, but we adopted a system in which the animals were backed up in the squeeze chute (Cummings and Son, Garden City, KS) such that the head-gate closed directly behind the ears, restraining most head and neck movement. The breath collection process took approximately 90 s, which included a 30 s

collection time, and a 60 s analysis time. Between animals, each mask was disinfected with a chlorhexidine diacetate solution and the viral filter was replaced.

Occasionally, atmospheric nitric oxide (aNO) in the processing facility exceeded 20-ppb interfering with breath test results by artificially increasing measured eNO results. This was due primarily to the fact that equipment burning fossil fuels sporadically entered the other end of the facility as part of daily feedyard operation and feed mixing. When the equipment was removed from the facility, levels quickly returned to less than 300 ppt. To ensure that reported means were not influenced by aNO contamination, breath data with eNO levels > 1.7 ppb were not included in the analysis as discussed earlier. This was based on eliminating data beyond two standard deviations greater than the mean of eNO levels measured when aNO was confirmed to be less than 100 ppt.

The TDLAS instrument operated without difficulty from September 14, 2005 to September 29, 2005 (designated as first period on Figure 4.6). On September 30, 2005, a leak was noticed in the sample collection bag of the instrument. This allowed dilution with ambient air to occur and a reduction of measured $eCO_2$ levels occurred (second period). After repairing the leak on October 17, 2005, $eCO_2$ returned to previous concentrations. Figures 4.7 and 4.8, show resulting eNO and eCO2 concentrations as measured by the TDLAS instrument for all Control and Treated calves, respectively, along with eCO2 concentrations measured with the NDIR-CO2 sensor over all three periods. The reduction in the levels of TDLAS measured eCO2 and eNO in Period 2 are attributed to the dilution of the sample with ambient air as a result of the sample bag leak. If atmospheric $CO_2$ was diluting the sample during this period, aNO must have been diluting it as well. This supports the assumption that NO levels overall (with the

exception of times of fossil fuel burning, for which data were excluded from analysis) were primarily influenced by exhaled NO and not atmospheric NO. If aNO were dominant, we would not expect to see a significant drop in eNO during the 2nd Period. Therefore, since both Control and Treated calves were measured during all three periods, the data were analyzed together until proper corrections can be made.

No reports could be found in the literature in which eNO was measured in cattle. However, the few in vitro reports suggest that NO is produced in response to BRD pathogens in the lower airway (Yoo et al., 1996; Mason et al., 1996; Mason et al., 2000). The current objective of our breath collection technique was to target lower airway breath (Schubert et al., 2001; Roller et al., 2006). The numeric trends observed from the arrival data suggest that eNO may be predictive of BRD, but the fact that eNO values were near the lower detection limits of current instrumentation and occasional contamination by atmospheric nitric oxide contributed to the fact that few statistical differences were found in this study. The one observed difference in eNO was between control calves and calves at the time of first treatment. Only ten percent of steers were treated more than once, while nearly 40% were treated once. The inability to find a difference between control steers and steers treated the second or third time is most likely due to sample size and variability of the measurement, since only 36 animals fell into these categories.

The concentration of eNO in breath is dependent on many factors. Bernareggi and Cremona (1999) compiled reported measurements of eNO in both humans and animals obtained by several collection and analytical methods. They observed that even in measuring similar patients using apparently similar methodology, different reports have quite different results. For example, in normal humans, using off-line tidal

breathing, mean eNO concentrations ranged from 8.6 to 15.4 ppb. The coefficient of variation (CV) for these measurements ranged from approximately 8 to 52%. The CV among our measurements was much greater. The CV of the eNO concentrations collected upon arrival was 132%. During the trial period, multiple measurements were made on air that had passed through a nitric oxide scrubber. The mean of these measurements (n=43) was 1 ± 225 ppt. Ambient air measured during the trial was 63 ± 297 ppt (n=32). The extensive variation observed in this study is primarily resulting from the instrument. It is likely that the standard deviations would not be as great if it were not for the extremely low concentrations observed.

Differences in $eCO_2$ between calves treated for BRD and those never treated was observed upon arrival, but the values were inverted when control calves were compared with treated animals. These differences could be due to differences in metabolism or breathing patterns while in the chute. The BRD pathogens and immune response severely damage lung tissue (Confer et al., 1990; Griffin, 1996). *M. haemolytica*, especially, is associated with coagulative necrosis, fibrin, and edema. The toxins produced by the pathogen destroy the macrophages and neutrophils. These toxins also increase the inflammatory cascade common with this disease. Proteolytic enzymes and oxidants can be released resulting in excess fluid accumulation and cell damage. Decreased pulmonary function, caused by the disease, could prevent diffusion of $CO_2$ from the capillaries into the alveoli reducing its concentration in the exhaled breath of clinically morbid animals.

*Haptoglobin.* During the trial, an excess of 1,100 serum tubes were collected and stored for serum Hp analysis. However, for the tubes collected from calves upon arrival,

we chose to analyze samples collected from load two only (n=130). Load two had the highest incidence of BRD from all four loads (54% treated at least once, Figure 4.9). For samples collected from control and treated animals, we chose to analyze only those collected during days 1-15 from each load. This includes a majority of all control and treated animals during the receiving period. All the samples analyzed for Hp in both categories correspond with a valid breath test (i.e., was not excluded because of the criteria mentioned above).

The Hp results that we report herein do not show any significant differences between arrival Hp and eventual treatment for BRD. This is in contrast with Young et al. (1996) who reported that within ten days after sampling, the proportion of calves with signs of BRD increased with Hp concentration. Carter et al. (2002) and Berry et al. (2004) found that serum Hp levels on days 0 and 7 of a preconditioning period were correlated with number of eventual treatments with BRD. The fact that the present study did not show statistical differences could be attributed to the smaller sample size used. However, since our reported numeric trend is aligned with previous reports, significant differences may be expected with a greater number of animals. At the time of treatment, control steers had lower Hp concentration than treated steers similar to the observation of Berry et al. (2004). The Hp results do support our clinical observation of BRD and increased rectal temperature as a threshold for treatment, and thatthe calves we classified as suffering from BRD were in fact ill. This experiment investigated the use of eNO as an objective measure of respiratory health, while the current methods of BRD diagnosis are subjective (Galyean et al., 1999). In reports comparing the presence of lung lesions at slaughter with previous health records, lesions were present in lungs from animals that

were never treated and previously treated animals had no evidence of disease present on the tissues (Gardner et al., 1999; Wittum et al., 1996). Since serum Hp concentrations currently seem to be a the best method of objectively measuring the presence of respiratory disease, any breath data comparing treated vs. control calves can be discussed as such—morbid vs. healthy.

*Conclusions.* The study presented here showed that NO could be measured real time in the breath of cattle using TDLAS. The additional time and labor required for the breath collection and analysis is not negligible considering the volume of animals that pass through many commercial feedlot processing chutes daily. However, numeric trends indicate that eNO might be useful to diagnose and monitor BRD and respiratory health in cattle. Further improvements in instrumentation and more research in the use of TDLAS technology are needed before it can be incorporated into a commercial setting.

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Table 4.1. Ingredient and nutrient composition of diet fed to receiving calves.

| Ingredient | % of diet, DM basis | Nutrient Composition[c] | |
|---|---|---|---|
| Rolled corn | 40.37 | DM, % | 89.3 |
| Corn dried distiller's grains w/sol. | 10.00 | $NE_m$, Mcal/cwt | 77.6 |
| | | $NE_g$, Mcal/cwt | 49.3 |
| Alfalfa hay | 20.00 | TDN, % | 73.0 |
| Cottonseed hulls | 20.00 | CP, % | 14.5 |
| Soybean meal | 5.00 | DIP, % of CP | 52.4 |
| Cottonseed meal | 2.40 | ADF, % | 31.4 |
| Wheat middlings | 0.95 | NDF, % | 46.5 |
| Limestone | 0.90 | eNDF, % NDF | 52.9 |
| Dicalcium phosphate | 0.10 | Ash, % | 5.25 |
| Salt | 0.20 | Potassium, % | 0.94 |
| Availa zinc[a] | 0.060 | Calcium, % | 0.70 |
| Zinc sulfate | 0.002 | Phosphorus, % | 0.37 |
| Vitamin A, 66,139 IU/lb. | 0.003 | Magnesium, % | 0.23 |
| Vitamin E, 50% | 0.002 | Sulfur, % | 0.21 |
| Rumensin 80[b] | 0.016 | Cobalt, ppm | 0.25 |
| | | Copper, ppm | 9.73 |
| | | Iron, ppm | 187.9 |
| | | Manganese, ppm | 46.0 |
| | | Selenium, ppm | 0.26 |
| | | Zinc, ppm | 258.6 |

[a]Zinpro, Inc., Eden Prairie, MN.
[b]Elanco Animal Health, Indianapolis, IN.
[c]DM is listed on as as-fed basis. All others are expressed on a DM basis. DM, Ash, ADF, NDF, and CP analyzed. All others are based on diet formulation from NRC (1996).

Table 4.2. Steer performance and arrival Hp and exhaled compound least squares means ± SEM.

| | | No. of Treatments for BRD | | |
|---|---|---|---|---|
| Item | n[a] | 0 | 1 | >1 |
| Percent of Calves | 395 | 56.0 | 33.9 | 10.1 |
| Initial BW (kg) | 394 | 217.9 ± 10.6[b] | 215.9 ± 10.6[bc] | 212.6 ± 10.8[c] |
| Final BW (kg) | 390 | 272.2 ± 10.4[d] | 264.2 ± 10.4[e] | 250.8 ± 10.3[f] |
| ADG (kg/d) | 390 | 1.32 ± 0.12[d] | 1.17 ± 0.12[e] | 0.93 ± 0.13[f] |
| Arrival Hp (µg/mL) | 136 | 166.0 ± 64.5 | 254.3 ± 65.4 | 232.9 ± 118.9 |
| Arrival eNO (ppt) | 379 | 287.7 ± 45.6 | 308.7 ± 49.8 | 338.9 ± 77.5 |
| Arrival $eCO_2$[g] (%) | 379 | 2.30 ± 0.38[d] | 2.57 ± 0.38[ef] | 2.43 ± 0.40[df] |

[a]Number of observations used for each variable.
[bc]Means in a row without a common superscript differ ($P < 0.10$).
[def]Means in a row without a common superscript differ ($P < 0.05$).
[g]Capnostat 5, Respironics, Inc. Carlsbad, CA.

Table 4.3. Rectal temperature, Hp, eNO, $eCO_2$ concentration least squares means ± SEM from steers during antimicrobial treatment or control steers.

| Item | n[a] | Control | 1st Treatment | 2nd Treatment | 3rd Treatment | P-value of contrast[b] |
|---|---|---|---|---|---|---|
| Temperature (ºC) | 525 | 39.4 ± 0.1[c] | 40.6 ± 0.1[d] | 40.5 ± 0.1[d] | 39.9 ± 0.2[d] | <0.001 |
| Serum Hp (µg/mL) | 208 | 810 ± 46[c] | 1,303 ± 56[d] | 1,457 ± 136[d] | 1,560 ± 349[d] | <0.001 |
| eNO (ppt) | 286 | 368.3 ± 114.2[c] | 564.5 ± 116.6[d] | 369.4 ± 159.5[cd] | 462.2 ± 230.9[cd] | 0.29 |
| $eCO_2$ (%)[e] | 388 | 2.62 ± 0.07[b] | 2.04 ± 0.09[d] | 1.87 ± 0.16[d] | 2.19 ± 0.26[cd] | <0.001 |

[a]Number of observations used for each variable.
[b]Contrast of control steers vs. average of all treated steers.
[cd]Means in a row without a common superscript differ ($P < 0.05$).
[e]Capnostat 5, Respironics, Inc., Carlsbad, CA.

Figure 4.1 Diagram of breath collection apparatus.

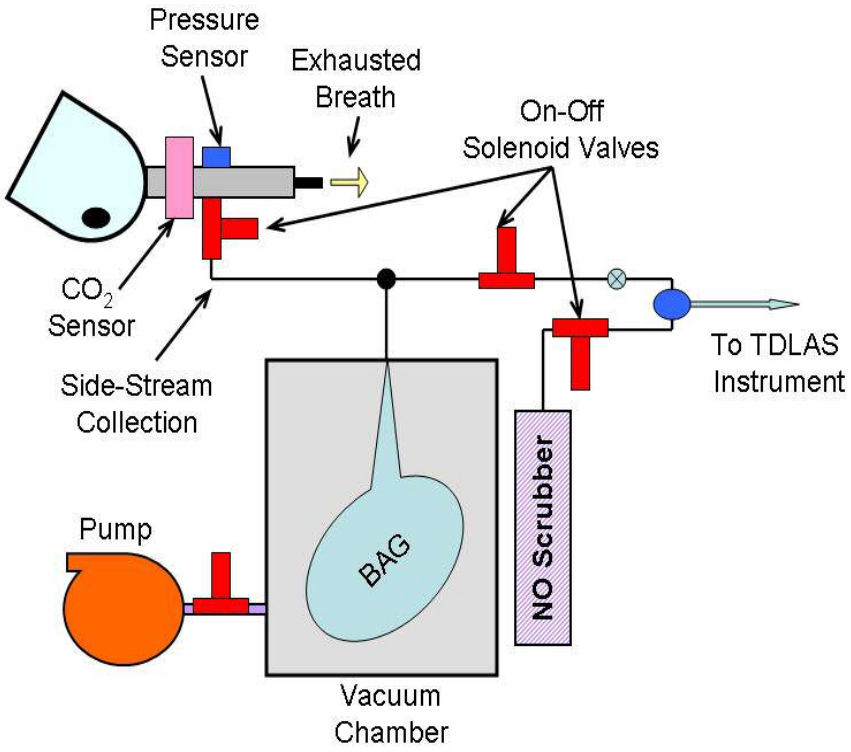


Figure 4.2. Carbon dioxide trend measured using the NDIR-capnograph and corresponding inhalation/exhalation pressure trend. The shaded regions indicate when the sample ON/OFF valve is open and collecting breath.

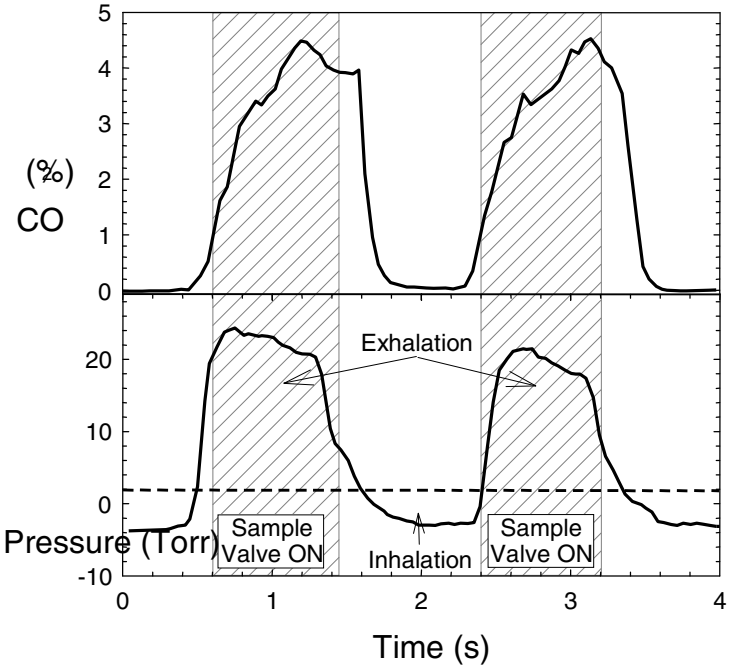


Figure 4.3. Acquired background, sample, and background subtracted spectra.

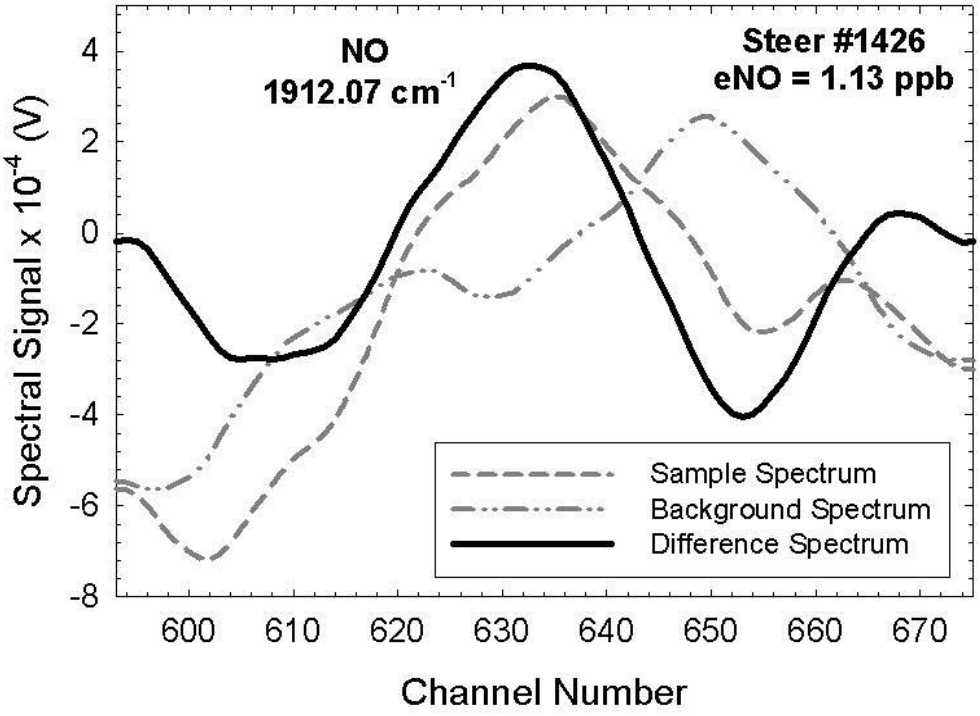


Figure 4.4. Photograph of an undergraduate employee collecting breath from a steer.

Figure 4.5. Photograph of collecting breath from a steer upon arrival showing breath collection (foregroung) and operation of the TDLAS instrument (background).

Figure 4.6. Exhaled carbon dioxide concentrations measured by the TDLAS instrument. The second period indicates the period in which the sampling bag had a leak.

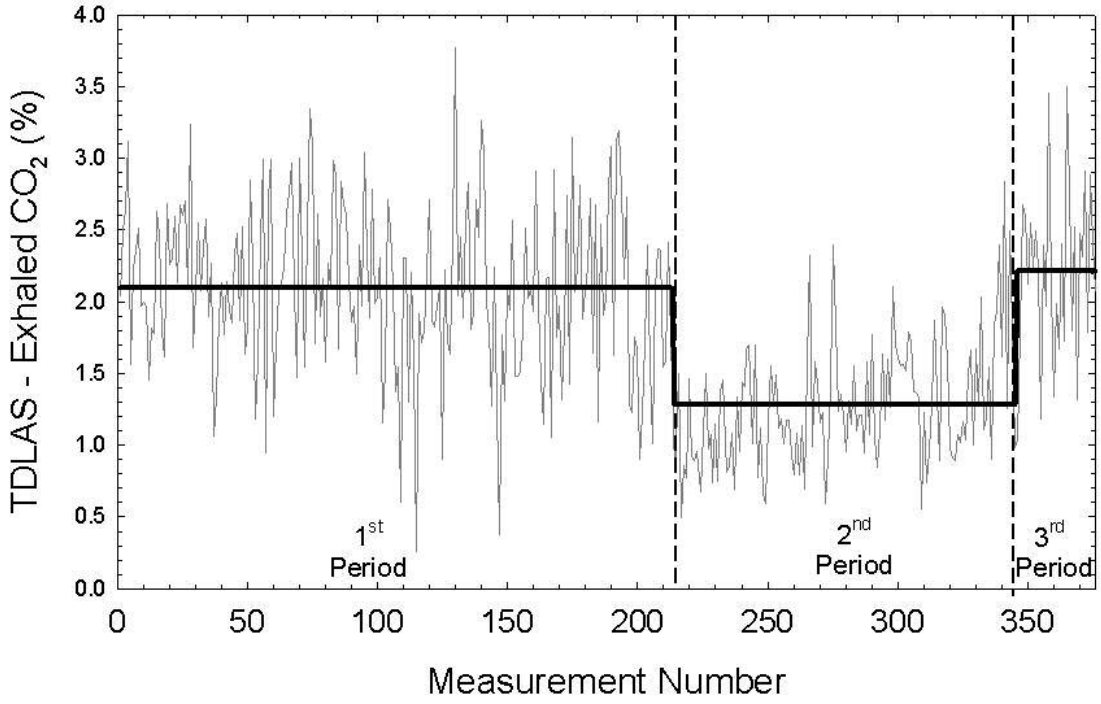


Figure 4.7. Bar plots of eNO concentrations for periods 1-3 identified in Figure 4.6.

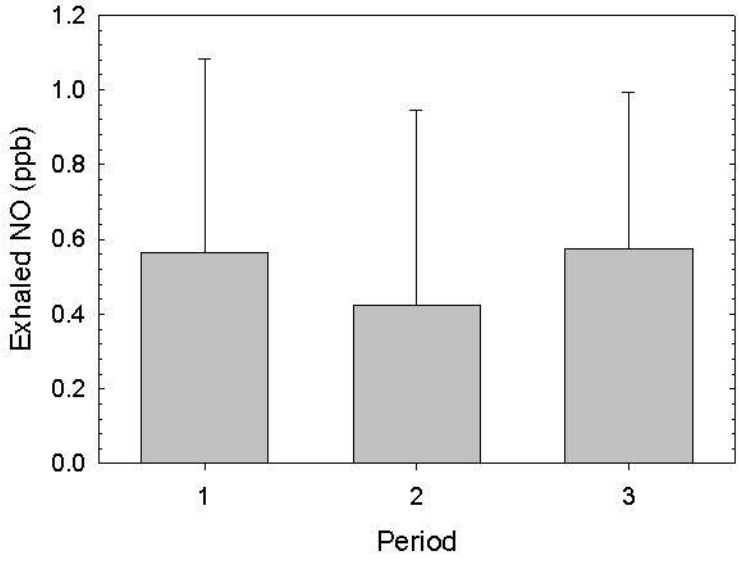



Figure 4.8. Exhaled carbon dioxide concentrations measured by the TDLAS instrument and the NDIR-capnograph sensor for the three periods.

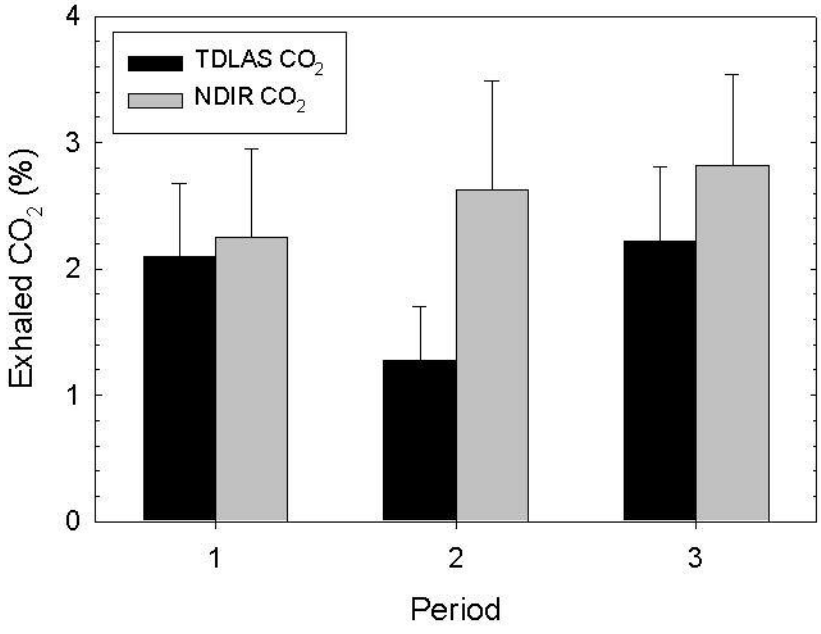
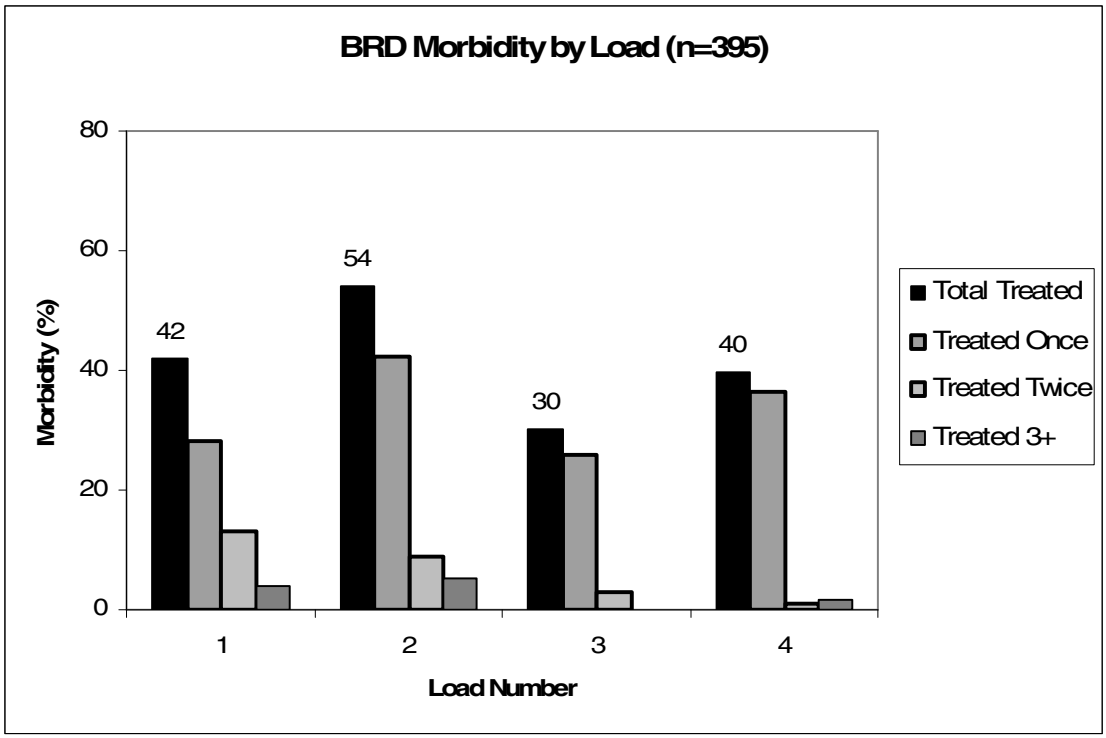


Figure 4.9. BRD morbidity by load.



VITA

Ben Patrick Holland

Candidate for the Degree of

Master of Science

Thesis: MEASUREMENT OF EXHALED NITRIC OXIDE AND EXHALED CARBON DIOXIDE IN THE BREATH OF BEEF CALVES

Major Field: Animal Science

Biographical:

Personal Data: Born in Dumas, Texas, on September 11, 1981, the son of John and Gail Holland.

Education: Graduated from Texline High School, Texline, Texas in May 2000; received Bachelor of Science in Animal Science from Texas Tech University, Lubbock, Texas in May 2004. Completed the requirements for the Master of Science degree with a major in Animal Science at Oklahoma State University in July, 2006.

Experience: Employed as a summer laborer by Mac Kehoe Cattle, Texline, Texas, 1995 to 2004; Department of Animal Science, Texas Tech University, Lubbock, Texas as an undergraduate research assistant, 2000 to 2002; graduate research assistant, 2004-present, and research coordinator, Willard Sparks Beef Research Center, 2005 to present, Department of Animal Science, Oklahoma State University.

Professional Membership: American Society of Animal Science

Name: Ben Patrick Holland Date of Degree: July, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: MEASUREMENT OF EXHALED NITRIC OXIDE AND CARBON DIOXIDE IN THE BREATH OF BEEF CALVES

Pages in Study: 62 Candidate for the Degree of Master of Science

ABSTRACT: Two experiments were conducted to test the ability to measure exhaled nitric oxide (eNO) and exhaled carbon dioxide ($eCO_2$) in the breath of healthy beef calves and calves showing clinical signs of bovine respiratory disease (BRD) using tunable diode laser absorption spectroscopy. In experiment one, three steers (avg. initial BW = 251.8 kg) were used in a preliminary study in which two steers were challenged intra-tracheally with *M. haemolytica*. Breath and blood, for serum Hp analysis, were collected as well as clinical responses recorded. Prior to the challenge, eNO levels in all three calves were approximately 1 ppb. The eNO levels increased to at least 2 ppb in the challenged steers. Experiment two used 395 steer and bull calves (avg. initial BW = 218.6 ± 22.4 kg) received from auction markets for breath measurements during a 42-d receiving trial. Upon arrival, all steers had breath sampled for eNO and $eCO_2$ analysis. Subsequently, all calves treated for signs of BRD were sampled as well as randomly selected clinically healthy control calves. Arrival mean eNO was 313.9 ± 415.2 ppt and mean $eCO_2$ was 2.64 ± 0.94%. No differences in eNO were found between cattle subsequently treated for BRD. Steers eventually treated for BRD exhaled higher levels of $CO_2$ than calves never treated ($P<0.05$). At the time of treatment, mean eNO was 368.3 ppt and 465.4 ppt for control and treated calves, respectively ($P=0.29$). Opposite of arrival data, $eCO_2$ was higher for control than treated calves (2.62 vs. 2.03%; $P<0.001$). Measurement of exhaled breath was successfully incorporated into the daily activities of a research facility, and numeric trends suggest eNO may be useful in diagnosing and monitoring BRD. However, the fact that eNO levels were near lower detection limits of current instrumentation and occasional contamination by high ambient NO decreased the accuracy of the measurement.

ADVISER'S APPROVAL: _____Clinton R. Krehbiel_____